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PATENT ABSTRACTS OF JAPAN

(11)Publication number : **57-007420**
(43)Date of publication of application : **14.01.1982**

(51)Int.CI. **A61K 35/74**

(21)Application number : **55-081471**
(22)Date of filing : **18.06.1980**

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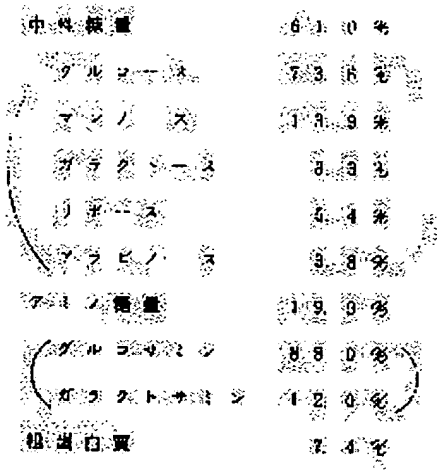
KODAMA KENJIRO

(54) ANTITUMOR AGENT

(57)Abstract:
PURPOSE: An antitumor agent comprising a component of the cell wall of a specific mold as an active ingredient.
CONSTITUTION: An antitumor agent comprising a component of the cell wall of a mold(e.g., "Aspergillus oryzae" F-1124, "Aspergillus tamarii" IFO4287, fungi belonging to Pleatscales, green mold, or the genus of "Aspergillus") belonging to the genus "Aspergillus" as an active ingredient. The cell wall is obtained by destroying the mold, washing it well, and removing the matter contained in it. For example, in "Aspergillus oryzae" F-1124, the component of the mold consists of the ingredients shown by the table. The antitumor agent is medicated by oral administration, injection, administration through the rectum, etc. Its acute toxicity LD50 is estimated to be ≥500mg/kg weight by oral administration and ≥200mg/kg weight by intra- abdominal administration.



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[Date of request for examination]

[Date of sending the examiner's decision of rejection]

[Kind of final disposal of application other than the examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

[Patent number]

[Date of registration]

[Number of appeal against examiner's decision of rejection]

[Date of requesting appeal against examiner's decision of rejection]

[Date of extinction of right]

(19) Japan Patent Office (JP)
(12) Laid-Open Patent Report

(11) Laid-open patent application no.
S57-7420
(43) Date laid open: January 14, 1982

(51) IPC: A 61 K 35/74
Identification code:
Internal control no.: 7138-4C

Inspection requested: No
Number of inventions: 1
(Total 5 pages)

(54) Antitumor agent

(21) Application no.: S55-81471
(22) Application date: June 18, 1980
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Specification

1. Title of Invention
Antitumor agent

2. Scope of Patent Claims
An antitumor agent that contains as an active ingredient a cell wall component of mold belonging to the *Aspergillus* genus.

3. Detailed Explanation of the Invention
This invention pertains to an antitumor agent that contains as an active ingredient a cell wall component of mold belonging to the *Aspergillus* genus.

In the past, several antitumor agents having fungus of microorganisms as an active ingredient have been reported, but all of them pertain to yeasts, bacilli or basidiomycetes, and the antitumor activity of fungus components of mold has not been reported.

As a result of various studies of the antitumor activity of the fungus components of mold, these inventors discovered that the cell wall component of mold belonging to the *Aspergillus* genus has antitumor activity, and they achieved this invention. That is, this invention offers an antitumor agent that contains as an active ingredient a cell wall component of mold belonging to the *Aspergillus* genus.

The "mold belonging to the *Aspergillus* genus" used in this invention is a microorganism belonging to asymmetric ascomycetes or the *Aspergillus* genus, whose cell wall components exhibit antitumor activity. Specific examples of molds belonging to the *Aspergillus* genus include *Aspergillus oryzae, Aspergillus sojae, Aspergillus tamarii, Aspergillus niger, Aspergillus usamii, Aspergillus saitoi, Aspergillus awamori, Aspergillus japonicus* and so forth. Among these, any strain that is compatible with the purpose of this invention can be selected.

The "cell wall component" that is the active ingredient of this invention is that prepared from the fungus of mold belonging to the *Aspergillus* genus. The cell wall is that which contains as a main ingredient a polysaccharide comprising at least glucose, mannose, galactose, N-acetyl glucosamine and so forth. As other sugar ingredients, the presence of ribose, arabinose, xylose,

laminaribese, glucosamine, galactosamine and so forth is known. For example, sample analysis values of the main composition of cell wall component prepared from *Aspergillus oryzae* F-1124 (Microorganism Laboratory fungus no. 1365) are shown below.

(Preparation method)

By nylon paste cultivation using soy bean–wheat as a culture medium, *Aspergillus oryzae* F-1124 was cultivated at 30°C for 72 hours, after which fungi were separated and removed from the top of the nylon cloth using a blade. The fungi were rinsed with deionized water, after which they were freeze-dried, and dried fungi were obtained. The dried fungi were pulverized by a dynomill, and they were stirred and extracted at 20°C for 3 hours using lauryl sodium sulfate. Then, they were stirred and extracted at 5°C for 16 hours, and the cell walls were centrifugally separated. After being rinsed, they were dialyzed and freeze-dried, and cell walls were thereby prepared.

(Analysis methods)

1) After the cell walls were hydrolyzed with sulfuric acid, neutral sugar was quantified by the Petri method, and this was expressed by glucose conversion.

2) After the cell walls were hydrolyzed with sulfuric acid, amino sugar was quantified by an amino acid automatic analyzer.

3) Crude protein was quantified by the Lowry-Folin method.

(Analysis values)

Neutral sugar quantity		61%
	Glucose	78.6%
	Mannose	13.9%
	Galactose	3.3%
	Ribose	5.4%
	Arabinose	3.8%
Amino sugar quantity		19.9%
	Glucosamine	88.0%
	Galactosamine	12.0%
Crude protein quantity		7.4%

The cultivation method of mold fungus to prepare the cell wall component, the separation method of the fungus, and the refinement method of the cell wall component are not particularly restricted. Known general methods can be suitably utilized, and processing methods for obtaining the suitable cell wall products can be devised appropriately depending on the purpose of this invention.

As the cultivation method of the mold fungus, any good cultivation methods can be used, such as ordinary liquid cultivation methods, and solid cultivation methods using agar, nylon paste, asbestos or sponge as the cultivation base, and mixed cultivation methods in which the two are combined. In the case of liquid cultivation methods, either the liquid surface cultivation method or in-liquid cultivation method can be used, and among in-liquid cultivation methods, the shaking cultivation method or deep cultivation method can be used. As the culture medium for liquid cultivation, natural cultivation bases such as yeast syrup and wheat germ syrup, and synthetic cultivation bases such as Zabeck liquid, Laurin liquid and Benneberg liquid can be given as examples, but any culture medium in which *Aspergillus* genus microorganisms can be used, and a culture medium that results in good fungus production can be selected. As the culture medium for solid cultivation, soybeans, wheat, barley, rice, bread, corn, fish powder, leaf protein, microorganism protein, gluten and so forth can be used individually or in combination. In addition, inorganic salts, vitamins, minerals and so forth can be added to these culture media, and any culture medium can be used as long as *Aspergillus* genus microorganisms can be grown under any culture medium conditions.

As cultivation conditions, optimal conditions can be selected in accordance with the culture medium composition, cultivation method and fungus strain, but as an example, in the case where *Aspergillus oryzae* F-1124 is cultivated by shaking cultivation in a glucose peptone culture medium, the fungi are produced with good yield under cultivation conditions of 28°C for 72 hours.

Fungus is separated and removed from the cultivation matter by ordinary methods, and a suitable separation method is selected depending on the fungus strain. For example, in the case of liquid cultivation, after cultivation is finished, centrifugal separation, filtration, tilting or pressure rods can be used.

The fungus is pulverized by physical methods using a homogenizer, dynomill, French press and so forth. To make pulverization easy, pretreatment by chemical methods, enzyme methods, organism methods and so forth can be performed in advance to an extent that does not harm the active ingredient. As chemical treatments, there are acid treatment by hydrochloric acid, sulfuric acid, acetic acid or citric acid, alkali treatment by sodium hydroxide, potassium hydroxide or calcium hydroxide, treatment by salts such as sodium chloride, or treatment by organic solvents such as alcohol, chloroform or acetone. After the fungus is immersed and stirred into each pharmaceutical solution, it is treated by heating and drying. In enzyme treatment, enzymes that do not harm the active ingredient are used to make pulverization of fungus easy, by cell wall softening, cell internal substance elution promotion and so forth. In physiochemical treatment, parts of animals or microorganism cells are used. For example, in the case of animals, gastric juices and intestinal juices are used, and in the case of microorganisms, fungus can be treated by immersion, stirring, heating or drying using protoplasts.

To prepare the cell walls from the pulverized fungus, elution of contents in the fungus is required. As the eluate, water, salt solutions (sodium chloride aqueous solution, potassium chloride aqueous solution and so forth), acids (trichloracetic acid, perchloric acid and so forth), alkalis (sodium hydroxide, potassium hydroxide and so forth), organic solvents (ether, hexane, methanol, chloroform and so forth), surface active agents (lauryl sodium sulfate, polyoxyethylene lauryl ether, glycerin fatty acid ester, sucrose fatty acid ester, sorbitan fatty acid ester and so forth) and so forth can be used individually, or in combinations of two or more. As for the treatment method and conditions for elution, any treatment method within a range that does not harm the active ingredient can be used, and no particular conditions are required. The simplest method is elution by stirring. In the case of elution by stirring, stirring and extraction for 3-6 hours at room temperature is sufficient. A cell wall component is obtained by centrifugal separation or filtration of the obtained fungus suspension, and the eluate is completely removed by rinsing or dialysis, and cell walls are obtained.

The rinsed cell walls are normally dried. As the drying method, spray drying, air drying, freeze drying, vacuum drying and so forth can be used, as long as they do not harm the active ingredient. The yield of the cell wall component is 30-40% by dry conversion in the case of *Aspergillus oryzae* F-1124.

The cell wall component prepared in this way can be used as is as the active ingredient of this invention, but it is preferable to perform a sterilization process. As sterilization processes, it is possible to use boiling, evaporation, drying, irradiation, ultraviolet rays and so forth. Sterilization conditions can be ordinary conditions; for example, evaporation sterilization on something suspended in phosphoric acid buffer solution can be performed at 119°C for 10 minutes.

The antitumor agent of this invention can be administered orally, by injection, directly into the tumor, or by other suitable administration methods. The method of producing the agent suitable for the administration method is an ordinary method. That is, depending on the purpose, whether oral or injection, any suitable agent type can be decided upon. In the case where the cell walls are in powder form, a dispersed agent, a tablet, or a liquid agent in which the powder is dispersed in a liquid dispersant can be produced.

The dosage of the cell wall component should be determined by a physician while taking into consideration the source origin of the cell walls, the preparation method, administration method, disease condition and condition of the patient, but it is generally 1-50 mg/kg of body weight per day.

Next, the antitumor effect and acute toxicity of the cell wall component are described. Furthermore, the cell wall component used in these tests was obtained by the following preparation method.

100 ml of a glucose peptone culture medium comprised of 5% glucose, 0.5% polypeptone, 0.05% monopotassium phosphate, 0.05% dipotassium phosphate, 0.04% magnesium sulfate and 0.04% calcium chloride of pH 5.6 was put into a 500-ml flask, and after *Aspergillus* genus microorganisms were planted, they were cultivated for 72 hours at 28°C while being shaken. After cultivation was finished, the fungus was separated using a glass filter, it was rinsed with sufficient water and freeze-dried, and fungus was obtained. The fungus was pulverized for 60 minutes at 0°C in a dynomill, and then stirred and extracted at room temperature for 10 hours with 100 times its amount of 1% lauryl sodium sulfate. After extraction by stirring, it was centrifugally separated at 12,000 rpm for 30 minutes at 15°C. Then it was rinsed with deionized water, and dialysis with flowing water was performed at 5°C for 48 hours. It was freeze-dried, and a cell wall sample was prepared.

(1) Antitumor effect

Test example 1

Cell walls prepared from *Aspergillus oryzae* F-1124, *Aspergillus tamarii* IFO4287 and *Aspergillus sojae* IFO4274 were suspended in phosphate buffer solution containing 0.5% carboxymethylcellulose so as to result in 5.0 mg/ml and 2.5 mg/ml, and these were sterilized by heating for 10 minutes at 119°C, thereby creating agents.

2×10^7 cells of sarcoma 180 were transplanted under the skin on the rear of ICR-JCL mice (male, 7 weeks old), and 25 mg or 50 mg per kg of body weight of the agent was intraperitoneally administered a total of 9 times, on the 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 9^{th}, 11^{th}, 13^{th}, 15^{th} and 18^{th} day after transplantation. On the 32^{nd} day after administration was finished, tumor weight was measured. Mice in which absolutely no cancer tissue was seen were calculated as 0 g; mice in which traces were seen but were below the measurement limit of 0.1 g were all calculated as 0.1 g. These results are shown in Table 1.

Table 1

Tested agent	Dosage (mg/kg)	Tumor weight (g)	Average (g)	T/C (%)
Control	-	12.4, 12.0, 9.6, 9.2, 9.2, 9.1, 9.0, 8.4, 7.8, 7.2, 4.2, 4.1	8.5	100
Aspergillus oryzae F-1124 cell walls	25	0.5, 0.4, 0.2, 0.1, 0.1, 0.1, 0, 0, 0, 0, 0	0.18	1.5
	50	6.5, 1.9, 1.3, 0.4, 0.2, 0.1, 0, 0, 0, 0	1.04	12.2
Aspergillus tamarii IFO4287	25	9.5, 6.7, 0.4, 0.3, 0.1, 0.1, 0, 0, 0	1.90	22.4
	50	1.4, 0.1, 0.1, 0.1, 0.1, 0.1, 0	0.39	4.6
Aspergillus sojae IFO4274	25	6.0, 0.6, 0.1, 0, 0, 0, 0, 0, 0, 0	0.61	7.2
	50	2.2, 1.9, 1.1, 0.6, 0.4, 0.1, 0.1, 0, 0, 0	0.64	7.5

As a result of significance testing, there was a significant difference from the control group with $P < 0.005$ for all treated groups, and it was seen that there is a marked tumor growth prevention effect.

Test example 2

An antitumor effect test by oral administration was conducted as follows.

Cell walls of *Aspergillus oryzae* F-1124 were suspended in phosphate buffer solution in a proportion of 12.5 mg/ml, thereby creating an agent.

10^6 cells of sarcoma 180 were transplanted under the skin on the rear of ICR-JCL mice (female, 10 weeks old), and forced oral administration of 0.2 ml per 10 g of body weight of the mouse (25 mg/kg body weight) of the agent was performed 10 times, every other day starting 24 hours after transplantation. On the 4^{th} day after the last administration, tumor weight was measured. These results are shown in Table 2.

Table 2

Tested agent	Tumor weight (g)	Average (g)	T/C (%)
Control	30.3, 24.7, 19.0, 24.5, 10.4, 16.0, 14.4, 8.6, 1.2	16.6	100
Aspergillus oryzae F-1124 cell walls	20.5, 18.0, 15.2, 8.0, 6.7, 4.2	12.2	73

Test example 3

Cell walls of *Aspergillus tamarii* IFO4287 and *Aspergillus sojae* IFO4274 were suspended in sterilized physiological saline solution in a homogenizer so as to result in 4.0 mg/ml, this was diluted by half, then it was boiled, thereby producing an agent.

10^6 cells of sarcoma 180 were transplanted under the skin on the right thigh of ICR-JCL mice (female, 7 weeks old), and 0.05 ml per 10 g of mouse body weight was injected under the skin on the rear for a total of 10 doses, once every other day starting the day after transplantation. On the 87^{th} day after transplantation, the mice were anesthetized, cancer under the skin was cut out, and its weight was measured. These results are shown in Table 3.

Table 3

Tested agent	Tumor weight (g)	Average (g)	T/C (%)
Control	12.3, 8.0, 8.0, 17.2, 11.3, 15.0, 14.7	12.4	100
Aspergillus tamarii IFO4287	5.8, 5.7, 15.1, 14.2, 14.5, 14.7, 0	10.0	81
Aspergillus sojae IFO4274	0, 0, 0, 0, 0, 15.0	2.5	20

(2) Acute toxicity

Acute toxicity in mice was as follows.

Female ICR-JCL mice, 10 weeks old, body weight 30-32 g were used. The administration methods used were oral and intraperitoneal. The presence of death and general symptoms were observed for 7 days after administration of *Aspergillus oryzae* F-1124 cell walls. As a result, no cases of death were seen even at the maximum dosage that could be technically administered, and LD_{50} was more than 500 mg/kg body weight in oral administration, and more than 200 mg/kg body weight in intraperitoneal administration.

Patent Applicant: (677) Yamasa Shoyu Co., Ltd.

Formal Correction Form (voluntary)
March 30, 1981

To: Patent Office Director-General

1. Display of article
   Patent application no. S55-81471

2. Title of invention
   Antitumor agent

3. Party making correction
   Relationship to article: Patent applicant
   Address: 10-1 Niifucho 2-chome, Choshi City 288
   Name: (677) Yamasa Shoyu Co., Ltd.
   Representative: Y. Hamaguchi

4. Object of correction
   Detailed explanation of the invention section of Specification

5. Contents of correction
   1) On the last line of page 2 of the Specification, insert "indicates a component of cell walls that has antitumor effect, that" between "The "cell wall component" that is the active ingredient of this invention is one that" and "is prepared from the fungus of mold belonging to the *Aspergillus* genus."
   2) On the second line of page 9 of the Specification, correct "The cell wall component prepared in this way can be used as is as the active ingredient of this invention, but it is preferable to perform a sterilization process." to "The cell wall component prepared in this way can be used as is as the active ingredient of this invention, but it can also be used after refining the active ingredient further from the cell walls. When administering this cell wall component, it is preferable to perform a sterilization process."
   3) On page 10 of the Specification, insert the following text between the 19th and 20th lines (before (1) Antitumor effect).
   "Also, in test example 4, the cell walls were extracted for 3 hours by 1N sodium hydroxide, and this was repeated 3 times, then centrifugal separation was performed at 5°C for 20 minutes at 12,000 rpm, and the supernatant was neutralized to pH 7.0 with acetic acid. Centrifugal separation was performed again, and the residue was re-suspended in deionized water, and dialysis with flowing water was performed for 48 hours at 5°C, then it was freeze-dried, and an active ingredient sample was produced."
   4) On page 14 of the Specification, insert the following text after the last line.
   "Test example 4

   An active ingredient sample of *Aspergillus oryzae* F-1124 was suspended in phosphate buffer solution containing 0.5% carboxymethylcellulose so as to result in 2.5 mg/ml, and this was sterilized by heating at 119°C for 10 minutes, thereby producing an agent.

   2×10^7 cells of sarcoma 180 were transplanted under the skin on the rear of ICR-JCL mice (male, 7 weeks old), and 25 mg per kg of body weight of the agent was intraperitoneally administered a total of 10 times on the 1st day after transplantation. On the 30th day after administration, tumor weight was

measured. T/C (%) was 2.5%, and the total tumor disappearance ratio was 80%."

☐ Generate Collection

L13: Entry 1 of 2 File: JPAB Jan 14, 1982

PUB-NO: JP357007420A
DOCUMENT-IDENTIFIER: JP 57007420 A
TITLE: ANTITUMOR AGENT

PUBN-DATE: January 14, 1982

INVENTOR-INFORMATION:
NAME COUNTRY
SASAKI, MASAHARU
KODAMA, KENJIRO

ASSIGNEE-INFORMATION:
NAME COUNTRY
YAMASA SHOYU CO LTD

APPL-NO: JP55081471
APPL-DATE: June 18, 1980

INT-CL (IPC): A61K 35/74

ABSTRACT:

PURPOSE: An antitumor agent comprising a component of the cell wall of a specific mold as an active ingredient.

CONSTITUTION: An antitumor agent comprising a component of the cell wall of a mold(e.g., "Aspergillus oryzae" F-1124, "Aspergillus tamarii" IFO4287, fungi belonging to Pleatscales, green mold, or the genus of "Aspergillus") belonging to the genus "Aspergillus" as an active ingredient. The cell wall is obtained by destroying the mold, washing it well, and removing the matter contained in it. For example, in "Aspergillus oryzae" F-1124, the component of the mold consists of the ingredients shown by the table. The antitumor agent is medicated by oral administration, injection, administration through the rectum, etc. Its acute toxicity LD50 is estimated to be ≥500mg/kg weight by oral administration and ≥200mg/kg weight by intra- abdominal administration.

End of Result Set

Generate Collection

L13: Entry 2 of 2 File: DWPI Jan 14, 1982

DERWENT-ACC-NO: 1982-14619E
DERWENT-WEEK: 198208

TITLE: Antitumoural drug - contains cell wall component of fungi of genus aspergillus

PATENT-ASSIGNEE:

ASSIGNEE	CODE
YAMASA SHOYU KK	YAMS

PRIORITY-DATA: 1980JP-0081471 (June 18, 1980)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
JP 57007420 A	January 14, 1982		005	

INT-CL (IPC): A61K 35/74

ABSTRACTED-PUB-NO: JP 57007420A
BASIC-ABSTRACT:

Antitumoural drug contains cell wall component of fungi of genus Aspergillus as active component. The active component inhibits growth of tumours, e.g. Sarcoma 180. Acute toxicity (LD50) of the active component in mice is more than 500 mg/kg by oral admin. and more than 200 mg/kg by abdominal admin.

Examples of fungi are A. oryzae, A. sojae, A. tamarii, A. niger, A. usamii, A. saitoi, A. awamori, A. japonicus. The cell wall component contains as polysaccharide consisting of glucose, mannose, galactose and N-acetylglucosamine. Cultivation is conducted aerobically.

TITLE-TERMS: ANTITUMOUR DRUG CONTAIN CELL WALL COMPONENT FUNGUS GENUS ASPERGILLUS

DERWENT-CLASS: B04 D16

CPI-CODES: B04-B02B; B12-G07; D05-C;

CHEMICAL-CODES:

Chemical Indexing M1 *01*
Fragmentation Code
M423 M781 M903 P633 Q233 V500 V550

⑲ 日本国特許庁 (JP)　　⑪ 特許出願公開

⑫ 公開特許公報 (A)　　昭57—7420

⑤1Int. Cl.³　識別記号　庁内整理番号
A 61 K 35/74　7138—4C

⑬公開 昭和57年(1982) 1月14日

発明の数 1
審査請求 未請求

(全 5 頁)

⑤4抗腫瘍剤

⑳特願 昭55—81471
㉒出願 昭55(1980) 6月18日

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明細書

1. 発明の名称

抗腫瘍剤

2. 特許請求の範囲

アスペルギルス属に属するカビの細胞壁成分を活性成分として含有する抗腫瘍剤。

3. 発明の詳細な説明

本発明は、カビの細胞壁成分を活性成分とする抗腫瘍剤に関するものである。

従来、微生物の菌体成分を活性成分とする抗腫瘍剤がいくつか報告されているが、いずれも酵母、細菌もしくは担子菌に関するものであり、カビの菌体成分の抗腫瘍活性については報告されていない。

本発明者らはカビの菌体成分の抗腫瘍性について種々検討した結果、アスペルギルス属に属するカビの細胞壁成分が抗腫瘍性を有することを見い出し、本発明を完成するに至つた。すなわち、本発明は、アスペルギルス属に属するカビの細胞壁成分を活性成分として含有する抗腫瘍剤を提供するものである。

本発明において用いられる「アスペルギルス属に属するカビ」とは、不整子のう菌類、コウジカビ目、アスペルギルス属に属する微生物であつて、その細胞壁成分が抗腫瘍活性を示すものを称するものである。アスペルギルス属に属するカビの具体例としては、アスペルギルス・オリゼー(*Aspergillus oryzae*)、アスペルギルス・ソイエ(*A. sojae*)、アスペルギルス・タマリ(*A. tamarii*)、アスペルギルス・ニガー(*A. niger*)、アスペルギルス・ウサミ(*A. usamii*)、アスペルギルス・サイトイ(*A. saitoi*)、アスペルギルス・アワモリ(*A. awamori*)、アスペルギルス・ヤポニカス(*A. japonicus*)などが例示され、これらのうちから本発明の目的に適合する任意の菌株を選択すればよい。

本発明の活性成分である「細胞壁成分」は、前記アスペルギルス属に属するカビの菌体から調製されるものである。細胞壁は、少なくともグルコ

ース、マンノース、ガラクトース、N－アセチルグルコサミンなどから多糖体を主成分とするものである。その他糖成分としてはリボース、アラビノース、キシロース、ラミナリビース、グルコサミン、ガラクトサミンなどの存在が知られている。たとえばアスペルギルス・オリゼ－F－1124（微工研菌寄第1365号）から調製された細胞壁成分の主な組成の分析値の一例を次に示す。

〔調製法〕

アスペルギルス・オリゼ－F－1124を、大豆－小麦を培地としたナイロン－ペースト培養法により30℃、72時間培養した後、ナイロン濾布上の菌体を刃物を用いて分離取得した。菌体を脱イオン水で水洗後、凍結乾燥して乾燥菌体とした。乾燥菌体をダイノミルで菌体破壊後、1%ラウリル硫酸ナトリウムを用いて20℃、3時間撹拌抽出後、次いで5℃で16時間撹拌抽出し、細胞壁を遠心分離し、水洗後、透析し、凍結乾燥して細胞壁を調製した。

〔分析法〕

1) 中性糖量は、細胞壁を硫酸で加水分解後、シャーレス法で定量し、グルコース換算で示した。

2) アミノ糖は、細胞壁を塩酸で加水分解後、アミノ酸自動分析計で定量した。

3) 粗蛋白質は、ロウリー－フォリン（Lowry－Folin）法で定量した。

〔分析値〕

中性糖量	61.0%
（グルコース	73.6%）
（マンノース	13.9%）
（ガラクトース	3.3%）
（リボース	5.4%）
（アラビノース	3.8%）
アミノ糖量	19.9%
（グルコサミン	88.0%）
（ガラクトサミン	12.0%）
粗蛋白質	7.4%

細胞壁成分を調製するためのカビの菌体の培養法、菌体の分離取得法、細胞壁成分の精製法には特に制約されない。公知の一般法が適宜に採用されうるし、本発明の目的により適した細胞壁標品を得るための処理法を適宜に工夫しうる。

カビの菌体の培養法は、通常の液体培養法、カンテン、ナイロン－ペースト、アスベストもしくはスポンジなどを培養基とする固体培養法、および両者を組み合わせた混合培養法などいずれの好気的培養方法をも適用できる。液体培養法の場合は、液面培養法、液内培養法のいずれでもよく、液内培養法では振盪培養法、深部培養法などの方法を採用しうる。液体培養のための培地としては、たとえば麹汁、麦芽汁などの天然培養基、ツアペック液、ラウリン液、ヘンネベルグ液などの合成培養基が例示できるが、アスペルギルス属微生物が生育しうる培地であればよく、菌体生産に好適な培地を適宜に選択できる。固体培養のための培地としては、たとえば大豆、小麦、小麦麩、米粒、パン、トウモロコシ、魚粉、葉蛋白、微生物蛋白、グルテンなどが単独であるいは組合せて用いられる。さらにこれらの培地に無機塩、ビタミン、ミネラルなどを添加したものでも勿論よく、いずれの培地条件でもアスペルギルス属微生物が生育しうる培地であれば適用できる。

培養条件は培地組成、培養方法、菌株に応じて適宜に最適な条件を選択すればよいが、一例を示せば、アスペルギルス・オリゼ－F－1124をグルコース－ペプトン培地で振盪培養した場合、28℃、72時間の培養条件で菌体が収量よく生産された。

菌体は培養物から常法により分離取得され、培養条件、菌株に応じて適切な分離取得法が選択される。たとえば、液体培養法の場合は、培養終了後、遠心分離法、濾過法、傾斜法、圧搾法などが採用される。

菌体をホモゲナイザー、ダイノミル、フレンチプレスなどを用いる物理的方法で破壊する。破壊を容易にするために、活性成分を損失させない範囲であらかじめ化学的方法、酵素的方法、生物的方法などにより前処理を行ってもよい。化学的処理としては、塩酸、硫酸、酢酸、クエン酸などによる酸処理、水酸化ナトリウム、水酸化カリウム、

炭酸カルシウムなどによるアルカリ処理、塩化ナトリウムなどの塩による処理、アルコール、クロロホルム、アセトンなどの有機溶媒による処理などがある。各薬剤溶液に菌体を浸漬し、撹拌した後、加熱、乾燥することにより処理される。酵素的処理では、細胞壁軟化、細胞内物質溶出促進など菌体破壊を容易にし、活性成分を損失させない酵素が用いられ、動植物あるいは微生物などその起源は問わない。生物学的処理では動植物あるいは微生物細胞の一部が用いられる。たとえば動物の場合、胃液、腸液など、微生物の場合はプロトプラストを用いて菌体を浸漬、撹拌、加熱、乾燥処理すればよい。

破壊菌体から細胞壁を調製するためには菌体内容物を溶出することが必要である。溶出剤としては、水、塩溶液（食塩水、塩化カリウム水溶液など）、酸（トリクロル酢酸、過塩素酸など）、アルカリ（水酸化ナトリウム、水酸化カリウムなど）、有機溶媒（エーテル、ヘキサン、メタノール、クロロホルムなど）、界面活性剤（ラウリル硫酸ナトリウム、ポリオキシエチレンラウリルエーテル、グリセリン脂肪酸エステル、しよ糖脂肪酸エステル、ソルビタン脂肪酸エステルなど）などの一種がまたはこれらの二種以上が組み合わせられて用いられる。溶出にあたつての処理方法、条件は、活性成分を損失させない範囲で処理すればよく、特別の方法条件を必要としない。最も簡単には撹拌溶出がよい。撹拌溶出の場合は、室温で3～6時間撹拌抽出すれば十分である。かくして得られる菌体懸濁液を遠心分離または濾過処理することにより細胞壁画分を取得し、水洗もしくは透析などで溶出剤を完全に除去して、細胞壁を得る。

洗浄された細胞壁は通常乾燥する。乾燥方法は活性成分を損失させない範囲ならば、噴霧乾燥、通風乾燥、凍結乾燥、真空乾燥などいずれの方法でもよい。細胞壁成分の収率はたとえばアスペルギルス・オリゼーF－1124の場合、乾燥物換算で30～40％重量である。

このようにして調製された細胞壁画分をそのまま本発明の活性成分として供することができるが、滅菌処理を施した方が好ましい。滅菌処理法は、通常の方法でよく、たとえば煮沸法、蒸気法、乾熱法、放射線法、紫外線法などの方法が採用される。滅菌条件も通常の条件でよく、たとえばりん酸緩衝液に懸濁させたものに対しては蒸気滅菌で119°C、10分間処理でよい。

本発明の抗腫瘍剤は、経口、注射、経直腸その他合目的的な投与方法により投与することができる。その投与方法に好ましい剤型に製剤化する方法は常法による。すなわち、経口用、注射用等の目的によつてそれぞれ合目的的な任意の剤型を決定すればよい。細胞壁が粉末である場合には散剤、適当な賦形剤による錠剤、液体分散媒に分散させた液剤などに製剤化しうる。

細胞壁成分の投与量は、細胞壁の起源由来、調製形態、投与方法、病状、患者の状態その他を考慮して医師が決定すべき事項であるが、一般的にいえば1日当り1～50㎎／㎏体重程度である。

次に細胞壁成分の抗腫瘍作用、急性毒性について記述する。なお、これらの試験において用いられた細胞壁成分は次のような調製方法によつて得られたものである。

グルコース5％、ポリペプトン0.5％、りん酸1カリウム0.05％、りん酸2カリウム0.05％、硫酸マグネシウム0.04％、塩化カルシウム0.04％、pH 5.6よりなるグルコース・ペプトン培地100㎖を500㎖容坂口フラスコに入れ、アスペルギルス属微生物を植菌後、28°Cで72時間振盪培養した。培養終了後、ガラスフイルターで菌体を分離し、十分水で洗浄し、凍結乾燥して菌体を得た。菌体をダイノミルで0°C、60分間磨砕後、1％ラウリル硫酸ナトリウム100倍容で10時間、室温で撹拌抽出した。撹拌抽出後、12,000 r.p.m.、30分間、15°Cで遠心分離し、脱イオン水で水洗後、5°C、48時間流水透析を行い、凍結乾燥して細胞壁サンプルを調製した。

〔1〕 抗腫瘍作用

試験例　1

アスペルギルス・オリゼーF－1124、アスペルギルス・タマリIFO4287、アスペルギルス・ソイエIFO4274から調製された細胞壁を5.0㎎／㎖、2.5㎎／㎖になるように0.5％カルボキシメチルセルロース含有りん酸緩衝液に懸濁し、119℃、10分間加熱滅菌して製剤とした。

ICR－JCLマウス（雄、7週齢）にザルコーマ180細胞2×10^7個を背側皮下に移植し、移植後1日目、3日目、5日目、7日目、9日目、11日目、13日目、15日目、18日目の計9回体重1㎏当り25㎎、50㎎の薬剤を腹腔内投与した。投与終了後82日目に腫瘍重量を測定した。癌組織が全く認められないものを0gとし、痕跡程度認められるが、測定限界0.1g以下については全て0.1gとして計算した。これらの結果を第1表に示す。

第1表

試料	投与量(㎎／㎏)	腫瘍重量(g)	平均(g)	T/C(%)
対照	－	12.4, 12.0, 9.6, 9.2 9.2, 9.1, 9.0, 8.4 7.8, 7.2, 4.2, 4.1	8.50	100
アスペルギルス・オリゼー F-1124 細胞壁	25	0.5, 0.4, 0.2, 0.1 0.1, 0.1, 0. 0, 0, 0, 0,	0.13	1.5
	50	6.5, 1.9, 1.3, 0.4 0.2, 0.1, 0, 0, 0, 0	1.04	12.2
アスペルギルス・タマリ IFO 4287 細胞壁	25	9.5, 6.7, 0.4, 0.3 0.1, 0.1, 0, 0, 0	1.90	22.4
	50	1.4, 0.1, 0.1, 0.1 0.1, 0.1, 0	0.39	4.6
アスペルギルス・ソイエIFO4274 細胞壁	25	6.0, 0.6, 0.1, 0 0, 0, 0, 0 0, 0	0.61	7.2
	50	2.2, 1.9, 1.1, 0.6 0.4, 0.1, 0.1, 0 0, 0	0.64	7.5

有意差検定の結果、いずれの処理群でもP＜0.005において対照群と有意差が認められ、顕著な腫瘍発育阻止効果のあることがわかつた。

試験例　2

経口投与による抗腫瘍作用試験を次のように行つた。

アスペルギルス・オリゼーF－1124の細胞壁をりん酸緩衝液に12.5㎎／㎖の割合で懸濁させて製剤とした。

ICR－JCLマウス（雌、10週齢）にザルコーマ180細胞を10^6個背側皮下に移植し、移植後24時間目より隔日10回薬剤をマウス体重10g当り0.2㎖（25㎎／㎏体重）あて強制的に経口投与した。最終投与後4日目にマウスを殺し、腫瘍重量を測定した。この結果を第2表に示す。

第　2　表

試料	腫瘍重量(g)	平均(g)	T/C(%)
対照	30.3, 24.7, 19.0, 24.5 10.4, 16.0, 14.4, 8.6 1.2	16.6	100
アスペルギルス・オリゼーF-1124 細胞壁	20.5, 18.0, 15.2, 8.0 6.7, 4.2	12.2	73

試験例　3

アスペルギルス・タマリIFO4287、アスペルギルス・ソイエIFO4274の細胞壁をホモジナイザーで滅菌生理食塩水に4.0㎎／㎖になるように懸濁させ、1／2に希釈し、煮沸処理して製剤とした。

ICR－JCLマウス（雌、7週齢）にザルコーマ180細胞10^6個／0.1㎖を右大腿皮下に移植し、翌日より1日1回、隔日に計10回薬剤をマウス体重10g当り0.05㎖を背側皮下に注射した。移植後87日目にマウスを麻酔し、皮下の癌を切り出して重量を測定した。その結果を第3表に示す。

第　3　表

試料	腫瘍重量(g)	平均(g)	T/C(%)
対照	12.3, 8.0, 8.0, 17.2 11.3, 15.0, 14.7	12.4	100
アスペルギルス・タマリIFO 4287 細胞壁	5.8, 5.7, 15.1, 14.2 14.5, 14.7, 0	10.0	81
アスペルギルス・ソイエIFO 4274 細胞壁	0, 0, 0, 0, 0, 15.0	2.5	20

（Ⅰ）急性毒性

マウスにおける急性毒性は下記のとおりである。

マウスはＩＣＲ－ＪＣＬ系雌性、１０週齢 体重３０～３２９のものを用いた。投与系路は経口および腹腔内投与の二経路であつた。アスペルギルス・オリゼ－Ｆ－１１２４の細胞壁投与後７日間にわたつて死亡の有無ならびに一般症状の観察を行つた。その結果、技術的に投与可能な最大投与量においても全く死亡例は認められず、LD_{50}は経口投与において５００㎎／㎏体重以上、腹腔内投与において２００㎎／㎏体重以上と推定された。

特許出願人　（６７７）ヤマサ醤油株式会社

手続補正書（自発）

昭和５６年３月３０日

特許庁長官　島田春樹　殿

1. 事件の表示

昭和５５年特許願第８１４７１号

2. 発明の名称

抗腫瘍剤

3. 補正をする者

事件との関係　特許出願人

住所　郵便番号　２８８

千葉県銚子市新生町２丁目１０番地の１

名称　（６７７）ヤマサ醤油株式会社

代表者　濱口儀兵衛

4. 補正の対象

明細書の発明の詳細な説明の欄

5. 補正の内容

1) 明細書第２頁末行目の「される」と「ものである。」との間に「細胞壁の抗腫瘍性を有する成分を指称する」を加入する。

2) 明細書第９頁第２行目の「供することができるが、」を「供しうるが、細胞壁からさらに活性成分を分画、精製して用いることもできる。これらの細胞壁成分を投与するにあたつては」に訂正する。

3) 明細書第１０頁の第１９行目と第２０行目との間に次文を加入する。

「また試験例４では細胞壁に対して１Ｎ水酸化ナトリウムによる３時間抽出処理を３回繰り返し、１２,０００r.p.m.、２０分、５℃で遠心分離し、上澄液を酢酸でpH 7.0に中和した。再び遠心分離して残渣を脱イオン水に再懸濁させ、５℃、４８時間流れ透析を行ない、凍結乾燥して活性画分サンプルとした。」

4) 明細書第１４頁の末行目に続けて次文を加入する。

「試験例　４

アスペルギルス・オリゼ－Ｆ－１１２４の活性画分サンプルを2.5㎎／㎖になるように0.5％カルボキシメチルセルロース含有りん酸緩衝液に懸濁し、１１９℃、１０分間加熱滅菌して製剤とした。

ＩＣＲ－ＪＣＬマウス（雄、7.週齢）にザルコーマ１８０細胞を2×10^7個背側皮下に植し、移植後１日おきに体重１㎏当り２５㎎の薬剤を腹腔内に計１０回投与した。

投与後３０日目に腫瘍重量を測定したところ、Ｔ／Ｃ（％）は2.5％であり、完全腫瘍消失率は８０％であつた。」